Tumor and Stem Cell Biology

p53 Binds to and Is Required for the Repression of Arf Tumor Suppressor by HDAC and Polycomb

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Abstract

The expression of tumor suppressor Arf is tightly repressed during normal cell growth at a young age and is activated by oncogenic insults, and during aging, results in p53 activation and cell-cycle arrest to prevent hyperproliferation. The mechanisms of both transcriptional repression and activation of Arf are not understood. We show that p53 binds to and represses Arf expression and that this repression requires the function of both histone deacetylases (HDAC) and polycomb group (PcG) proteins. Inactivation of p53 leads to increased Arf transcription in both mouse embryonic fibroblasts (MEF) cultured in vitro and in tissues and organs of p53 null mice. Activation of endogenous p53 enhances Arf repression, and reintroduction of p53 back into p53 null MEFs restores Arf repression. Both DNA binding and transactivation activities of p53 are required for Arf repression. We show that p53 is required for both HDAC and PcG to repress Arf expression. Bindings of both HDAC and PcG to Arf are disrupted by inactivation of p53 and can be restored in p53 null MEFs by the reintroduction of wild-type, but not mutant, p53. These results indicate that p53 recruits both HDAC and PcG to Arf locus to repress its expression, and this repression constitutes a second feedback loop in p53 regulation. Cancer Res; 71(7); 2781–92. ©2011 AACR.

Introduction

Transcription factor p53 mediates cellular response to a wide range of genotoxic and growth stresses including DNA damage and oncogenic insults (1). Activated p53 increases the expression of numerous genes and elicits 3 distinct types of cellular outcomes—temporary cell-cycle arrest, permanent cell senescence, and apoptotic cell death—to prevent damaged or stressed cells from continuing proliferation (2). It is generally believed that escape from the p53-mediated checkpoint pathway is a necessary step for the development of most, if not all, types of tumors (3, 4).

Much has been learned on the function of p53 in both senescence and apoptosis (5). Equally important is the regulation of p53 in causing temporary cell-cycle arrest. Unlike senescence or apoptosis, both of which are irreversible, a mechanism to inhibit activated p53 is critically important in releasing temporarily arrested cells to resume the cell cycle once the damage is repaired or stress is relieved. One such mechanism to reversibly regulate p53 is the feedback inhibition loop in which p53 activates the expression of its principle inhibitor (6–8), MDM2, which binds to and inhibits the function of p53 by both repressing the transactivating activity of p53 (9, 10), as well as targeting p53 for ubiquitin-mediated degradation (11–13).

The Arf-p16 locus, which is altered in an estimated 30% to 40% of human tumors, encodes 2 distinct tumor suppressor gene products, p16reff2 and p14ARF (p19 in mouse), via the use of separate promoters and alternative reading frames (14–16). Whereas p16reff2 binds to and inhibits cyclin D-dependent CDK4 and CDK6 to retain the growth suppressive activity of Rb family proteins (17), ARF binds to and antagonizes the activity of MDM2, thereby stabilizing and activating p53 (18–20). The ARF gene is expressed at a low level in normally growing young cells. Previous studies have linked 2 histone-modifying complexes, histone deacetylases (HDAC; refs. 21–23) and polycomb proteins (PcG; refs. 24–30), to the repression of Arf expression. How these 2 histone-modifying complexes, which do not recognize a specific DNA sequence, are recruited to Arf locus is not known.

ARF is activated by various oncogenic insults or during cell aging, leading to the notion that ARF mediates an oncogene checkpoint pathway to prevent oncogenic-stimulated cells from hyperproliferating (15, 31). The potent activity of ARF in binding to and inhibiting the function of MDM2 raises the question of how p53 is reversibly regulated in oncogenically
insulted cells where ARF, if continuously expressed, would prevent MDM2 from inhibiting p53 and thus disrupt the MDM2–p53 feedback regulatory loop. It was noted early on that in both human (19, 32) and mouse cells (33, 34), ARF expression exhibits a strong inverse correlation with the functional status of p53, suggesting a possible feedback repression of ARF expression by p53. The molecular mechanism underlying this feedback regulation is unknown and is the focus of this study.

Materials and Methods

Cell culture, Western analysis, and antibodies

Mouse embryonic fibroblasts (MEF) and 293T cells were cultured in DMEM (Dulbecco’s modified Eagle’s medium) with 10% FBS. Cells were lysed with RIPA (radioimmunoprecipitation assay) buffer. Antibodies to Bmi1 (F6; Upstate), Ring1B (ab-3832; Abcam), Suz12 (ab-12073; Abcam), Ezh2 (ab-3748; Abcam), cdk1, and actin (C-11; Santa Cruz) were purchased from Santa Cruz Biotechnology. Normal rabbit IgG (Neomarkers), normal mouse IgG (Neomarkers), and p16 (M-156; Santa Cruz), 3m-H3K27 (ab-6002; Abcam), Upstate), p53 (FL-393X; Santa Cruz), p19 (ab-3832; Abcam), Suz12 (ab-12073; Abcam), Ezh2 (ab-3748; Abcam), and histone H4 (06-598; Upstate) were purchased from Abcam, mouse p16 (M-156; Santa Cruz), 3m-H3K27 (ab-6002; Abcam), E2F3 (sc-878X; Santa Cruz), tubulin (Ab-2 DM1A; Neomarkers), normal mouse IgG (Neomarkers), normal rabbit IgG (Neomarkers), and actin (C-11; Santa Cruz) were purchased commercially.

Retroviral procedures

The retroviral vector expressing mouse Bmi1 and microRNA targeting 3’-untranslated region (UTR) of mouse p53 was provided by Dr. Ned Sharpless and Yizhou He, respectively. Human p53 cDNA was cloned into pBABE-puro retrovirus vector, and point mutations were made by site-directed mutagenesis and verified by DNA sequencing. Retroviruses encoding short hairpin RNA (shRNA) silencing mBmi1, mE2F3, mHdac1, and mHdac2 were constructed by ligating respective oligonucleotides (see details in Supplementary Materials and Methods) into a PMKO-puro vector. Detailed experimental procedures for retroviral production and infection are described in the work of Kotake and colleagues (30).

Quantitative reverse-transcriptase real-time PCR

Detailed protocol has been described in the work of Kotake and colleagues (30). Sequences of PCR primers are described in Supplementary Materials and Methods. Mean values and SDs were calculated from triplicates of 3 independent repeats.

ChiP assay

Chromatin immunoprecipitation (ChiP) analysis was performed as described in the work of Kotake and colleagues (30). PCR was performed using Platinum Taq polymerase (Invitrogen) and primers on mouse Arf locus (see more details in Supplementary Materials and Method). For ChiP-Q-PCR, purified DNA was added to a quantitative reverse-transcriptase real-time PCR (Q-RT-PCR) mixture that contained 1× SYBR Green PCR master mix and 150 mmol/L gene-specific primers. Assays were performed in triplicate on a 7900 HT sequence detection system. Mean values and SDs were calculated from triplicates of 3 different repeats.

Results

p53 represses Arf expression in vivo

Confirming previous observations (33, 35), we found that the steady-state level of Arf protein is significantly increased in p53-deficient MEFs (Fig. 1A). We also observed a clear increase of p16 protein level in p53−/− MEFs, which could be caused by the decrease of p21 expression and then a reduction in function of the Rb pathway, which collaborates with polycomb repressive complex (PRC) to repress p16 gene transcription in a feedback loop (30). A demonstration of Arf repression by p53 in vivo, however, has been lacking. Therefore, we dissected 3 pairs of age-matched (1 pair at 5 weeks of age and 2 at 8 weeks of age) wild-type (WT) and p53 null mice and determined the Arf expression in 8 different organs/tissues. This study demonstrated that Arf expression was significantly increased by p53 loss in 4 organs (muscle, kidney, heart, and lung), moderately in 2 (liver and spleen) and unchanged in 2 (thymus and testis; Fig. 1B). This result provides the first evidence demonstrating p53-dependent repression of Arf in vivo.

To exclude the possibility that Arf accumulation in p53 null MEFs was caused indirectly by other potential mutations and/or adaptive changes accumulated during multiple rounds of cell division, we transduced WT MEFs with a retrovirus expressing the type 16 papilloma virus–encoded E6 oncoprotein that binds to and targets the degradation of p53, or a microRNA retrovirus targeting 3’-UTR of mouse p53. Ectopic expression of E6 or miR-p53 resulted in a detectable increase of Arf protein and mRNA as early as 2 days after viral transduction and a continual increase of Arf (Fig. 1C and Supplementary Fig. SA and B). This result supports a direct role of p53 in the repression of Arf transcription and also suggests a continuous need of p53 to maintain the repression.

Nutlins are a group of small compounds that can bind MDM2 in the p53-binding pocket and disrupt the p53–MDM2 interaction, leading to p53 activation (36). We treated WT MEFs at passage 2 with Nutlin-3 (10 μmol/L) or dimethyl sulfoxide (DMSO) for over 24 hours and then examined Arf expression. Confirming the activation of p53, Nutlin-3 increased p21 mRNA in WT MEFs, but had no effect on p21 level in p53−/− MEFs. Nutlin-3 treatment enhanced Arf repression in WT MEFs within 24 hours, resulting in a time-dependent decrease of Arf expression by 60%, but no effect on Arf level in p53−/− MEFs despite the much higher Arf transcription (Fig. 1D). We thus conclude that p53 represses Arf at a level of transcriptional regulation through a mechanism that requires a direct and continuous role of p53.

p53 binds to Arf locus

That p53 is directly involved in Arf repression led us to determine whether p53 binds to the Arf locus. We performed ChiP analysis using a panel of 35 pairs of oligonucleotide primers that span 4-kb upstream and 4-kb downstream of the transcription start site of mouse Arf. We detected direct p53 binding to a region immediately upstream and downstream of
exon 1β (amplicons a, b, and c for regular PCR; corresponding amplicons A, B, and C for Q-PCR) of Arf (Fig. 2A). In contrast, there was very little binding of p53 to the p16 promoter or no p53 binding was detected by using p53/C0/C0 MEF as negative control in ChIP analysis. These results demonstrate that p53 affects the expression of Arf and p16 differently and indicate a direct role of p53 in the repression of Arf expression.

p53-mediated repression of Arf needs both transactivation and DNA binding activity

To provide further evidence supporting a direct role of p53 in Arf repression, we examined 4 p53 mutants in Arf repression, including 2 well-characterized p53 hot spot mutants—R175H, which grossly disrupts protein conformation of p53 and R273H, which retains native conformation of p53 but loses contact

Figure 1. p53 represses Arf expression. A, the steady-state levels of Arf and p16 proteins were determined in WT and p53−/− MEFs at passage 5 (p5) by immunoblotting. B, three pairs of age-matched WT and p53−/− mice were dissected and total RNA was extracted from 8 different organ/tissues. The level of Arf mRNA was determined by Q-RT-PCR. C, WT MEFs (p2) were infected with mock or E6-expressing retrovirus and selected by G418 treatment. The level of p53 and Arf proteins or mRNA was determined by immunoblotting or Q-RT-PCR. D, WT (p2) and p53−/− MEFs were treated with 1% DMSO or 10 μmol/L Nutlin-3 and the levels of Arf and p21 mRNA were determined by Q-RT-PCR.
with DNA (see a recent review on the genetic and biochemical properties of different p53 mutants; ref. 37). In addition, we also examined a double mutant—L22Q/W23S—which disrupts transcriptional activity of p53, as well as the binding with Mdm2 (38) and a hyperactive p53 mutant (H178Y) that could rescue the inactivated function of a common mutation, G245S (39).

p53/C0/C0 MEFs were transduced with a retrovirus expressing the WT and individual mutants of p53. As expected, the 3 functional inactivation mutants (p53L22Q/W23S, p53R175H and p53R273H) were expressed at high levels, whereas cells could only tolerate a much lower level expression of both WT p53 and hyperactive p53H178Y mutant (Fig. 2B). The activity of the WT and individual p53 mutants was functionally verified by examining the expression of p21 (Fig. 2D).

ChIP and Q-RT-PCR assay showed that p53H178Y bound to Arf stronger than the WT p53 and p53L22Q/W23S exhibited decreased Arf binding. Both p53R175H and p53R273H, however, bound very weakly to Arf (Fig. 2C). We then determined whether these p53 mutants could restore Arf repression in p53 null MEFs. Starting 5 days after viral transduction, both WT and the hyperactive p53H178Y mutant reduced the Arf mRNA level by 50% (Fig. 2D). In contrast, ectopic expression of p53L22Q/W23S, p53R175H, and p53R273H at very high levels had little effect in restoring the repression of Arf. Together, these
results demonstrate that p53 represses Arf by directly binding to the Arf locus and that both transactivity and DNA binding of p53 are essential to repress Arf expression.

**HDAC binds to and represses Arf and is recruited to mArf in a p53-dependent manner**

HDACs associate with many transcriptional repressive complexes and have also been reported to participate in the transcriptional repression of both human and mouse ARF genes (22, 23). To determine whether HDACs are involved in p53-mediated repression of Arf transcription, we treated WT MEFs with trichostatin A (TSA), an inhibitor of class I/II HDACs. A low concentration of TSA (0.1 µmol/L) increased mouse Arf mRNA level by more than 15-fold within 24 hours (Fig. 3A). TSA treatment resulted in a detectable Arf protein increase as early as 6 hours, peaking around 25 hours and lasting to as long as 60 hours (Fig. 3B). These results demonstrate a potent role of HDACs in the repression of mouse Arf transcription.

**Figure 3. HDAC1 binds to and represses Arf in a p53-dependent manner.** A, WT p3 MEFs were treated with 0.1 µmol/L TSA for 12 or 24 hours. The levels of Arf protein or mRNA were determined by immunoblotting or Q-RT-PCR. B, WT p3 MEFs were treated with 0.1 µmol/L TSA for 0 to 72 hours. Cell pellets were collected at indicated time and steady-state level of protein was determined by immunoblotting. C, binding of HDAC1 to Arf locus was examined in WT p2 MEFs by ChIP analysis. The amount of DNA immunoprecipitated by HDAC1 or rabbit IgG was expressed relative to the percentage of input DNA. D, WT MEFs were treated with 1% DMSO or 10 µmol/L Nutlin-3 and cells were collected after 16 hours. The binding of HDAC1 on Arf was determined by ChIP-Q-PCR with indicated primers. F, p53–/– MEFs were infected with mock or WT and mutant p53 retrovirus. Cells were selected by puromycin treatment, collected at 5 days postinfection, and analyzed for HDAC1 binding to Arf by ChIP-Q-PCR.
We next tested the binding of HDAC1 on Arf in WT MEFs. ChIP showed that HDAC1 directly binds to a region immediately upstream of the transcription starting site of Arf, whereas no binding of HDAC1 was detected on the nearby p16 locus (Fig. 3C). Importantly, the binding of HDAC1 to mouse Arf was greatly decreased, by more than 60% (for amplicon A) to 80% (amplicon B), in p53<sup>−/−</sup> MEFs (Fig. 3D) or in WT MEFs with p53 knocked down by microRNA (Supplementary Fig. SC), providing the first evidence linking HDAC-mediated repression of Arf transcription to the function of p53. Supporting a functional dependency of deacetylation of Arf promoter on p53 function, activation of p53 by Nutlin-3 in WT MEFs resulted in a 6-fold increase of HDAC1 binding to Arf (Fig. 3E) and reintroduction of WT p53, but not p53<sup>226E/W235</sup> and p53<sup>R273H</sup>, back into p53<sup>−/−</sup> MEFs restored the binding of HDAC1 to the Arf locus (Fig. 3F). Finally, we showed that H4 acetylation of the Arf promoter in p53<sup>−/−</sup> MEFs was 2-fold higher than that in WT MEFs (Fig. 3G). Together, these results demonstrate that HDAC1 directly binds to and deacetylates Arf in a p53-dependent manner.

**p53 is required for PRC to bind and repress Arf**

Previous studies have shown that oncogene Bmi1 promotes cell proliferation and extends the life span of fibroblasts, in part, through repressing both p16<sup>ink4a</sup> and p19<sup>ARF</sup> expression (24, 25). More recently, PcG proteins have been shown to directly bind and repress both p16 and ARF in mouse cells (29, 30). To determine the functional interplay between p53 and PcG in repressing Arf expression, we transduced both WT and p53<sup>−/−</sup> MEFs with a retrovirus expressing the Bmi1 gene and determined Arf expression. Nine days after retrovirus transduction (passage 5), both the steady-state level of the Arf protein and mRNA were decreased substantially in Bmi1-overexpressing WT MEFs (Fig. 4A). In contrast, although Arf is expressed at a much higher level in p53<sup>−/−</sup> MEFs, overexpression of Bmi1 did not significantly affect either Arf protein or mRNA level. To determine the specificity of the functional dependency of Bmi1-mediated repression of Arf on p53, we examined the expression of 2 classical Bmi1-repressive targets—HoxA9 and HoxC13 (40). Ectopic expression of Bmi1 was capable of repressing both HoxA9 and HoxC13 genes regardless of the p53 status (Fig. 4B). Together, these results demonstrate a specific functional dependency of Bmi1-mediated repression of Arf transcription on p53.

Bracken and colleagues previously found that the PRC can bind directly to the mouse Arf locus to repress its expression (29). To search for the mechanism of the p53 dependency of Arf repression by Bmi1, we examined the binding of Bmi1 and Ring1B, another component of PRC, to the Arf locus in WT and p53<sup>−/−</sup> MEFs. Notably, the bindings of both Bmi1 and Ring1B to the Arf locus were greatly decreased in p53<sup>−/−</sup> MEFs (Fig. 4C). Confirming the p53 dependency, binding of Ring1B to the Arf locus in WT MEFs was also greatly reduced after transduction with an E6-expressing or p53-targeting microRNA retrovirus (Fig. 4D and Supplementary Fig. SC) and conversely increased in WT MEFs when p53 was activated by Nutlin-3 (Fig. 4E). Together, these results demonstrate a requirement of p53 for the binding of PRC1 to Arf locus.

Previous studies on Hox gene silencing by PRC suggest a sequential model whereby PRC2-mediated H3K27 trimethylation facilitates the recruitment of PRC1, which causes H2A-K119 ubiquitylation to repress Hox gene expression (41, 42). We further analyzed the binding of PRC2 and H3K27 trimethylation on Arf and quantification showed a dramatic decrease of this repressive marker in p53<sup>−/−</sup> MEFs to less than 7% (Fig. 4F). Likewise, the binding of 2 components of PRC2, Ezh2 and Suz12, to the Arf locus were also substantially reduced in p53<sup>−/−</sup> MEFs to 29% and 6.7% for Ezh2 and to 43% and 18% for Suz12 of WT MEFs at sites A and C, respectively (Fig. 4G). Hence, the p53 function is required for both PRC1 and PRC2 to bind to the Arf locus.

**Both transactivity and DNA binding activities of p53 are required for PRC1 binding to Arf**

To further demonstrate the requirement of p53 in facilitating the binding of PRC1 to the Arf locus, we reintroduced p53 into p53<sup>−/−</sup> MEFs which partially restored the repression of both Arf protein and mRNA (Fig. 5A and B). The binding of Ring1B to Arf was also restored when p53 was ectopically expressed, resulting in a 2- to 3-fold and 4- to 7-fold increase of Ring1B binding to Arf in p53<sup>−/−</sup> MEFs after adenovirus- and retrovirus-mediated p53 expression, respectively (Fig. 5A and B). We then determined whether mutant p53 restored PcG binding to Arf. Both WT p53 and the hyperactive p53<sup>S175T</sup> mutant, but not 3 inactivated mutants, restored Ring1B binding to Arf, leading to a 2.4- and 1.9-fold increase, respectively (Fig. 5C). Together these results demonstrate that both the trans-activating and DNA binding activities of p53 are required for the binding of PcG to Arf.

**HDAC function is required for PcG to bind mouse Arf**

Given that p53 is required for the binding of both HDAC and PcG complexes to the Arf locus and that HDAC has been reported to be in the same repressive complex with and facilitates the repressive function of PcG (43, 44), we next determined the requirement of HDAC function for PcG binding on Arf by 2 different approaches, pharmacologic inhibition of HDAC activity and shRNA-mediated depletion of HDACs. Treatment of WT MEFs with TSA and another pharmacologic inhibitor of HDAC, sodium butyrate (NaB), both significantly increase Arf expression, but neither affected Ring1B level (Fig. 6B). Inhibition of HDAC by either TSA or NaB increased H4 acetylation, yet decreased Ring1B binding and H3K27 trimethylation on Arf promoter (Fig. 6A and C).

We then used shRNA to knockdown HDAC1 and HDAC2 in WT MEFs. Although only a partial depletion was achieved, we observed a decrease of Bmi1 binding to Arf and an increase of Arf expression (Fig. 6D). These results demonstrate that the activity of HDAC is required for PcG to bind to and repress the expression of Arf, and both HDAC1 and HDAC2 contribute to this inhibition.

**HDAC and PcG function are required for p53 to repress Arf expression**

The functional dependency of both HDAC and PcG on p53 in the repression of Arf expression led us to determine whether
these 2 histone-modifying enzymes are required for p53 to repress Arf expression. To this end, we inhibited endogenous HDAC with TSA in WT MEFs and then determined whether activation of endogenous p53 by Nutlin can still repress Arf. While activation of p53 by Nutlin-3 resulted in a greater than 50% decrease of Arf expression in control MEFs, it had no detectable effect on Arf expression when the HDACs were inhibited by TSA, even though the Arf mRNA level was elevated by as much as more than 16-fold in the TSA-treated cells (Fig. 7A). As a control for p53 activation, inhibition of HDACs by TSA did not affect the increase of p21 expression by Nutlin-activated p53.

To determine whether the function of PcG is required for p53 to repress Arf expression, we knocked down the expression of Bmi1 in WT MEFs and examined the effect of Bmi1 silencing on Arf repression by Nutlin-activated p53. All 3 shBmi1 viruses efficiently reduced the expression of Bmi1 (Fig. 7B), and none of them affected the activation of p21 expression by Nutlin-activated p53. While activation of p53 by Nutlin-3 effectively reduced Arf expression by more than 50%
in WT MEFs infected with shRNA control virus targeting luciferase, it had no significant effect to reduce Arf expression in MEFs transduced with any of the 3 shRNA virus targeting Bmi1. Hence, the function of p53 in the repression of Arf expression is also dependent on PcG.

Discussion

The results demonstrate a direct role of p53 in the repression of mouse Arf transcription by showing the direct binding of p53 to the mouse Arf locus and that p53 is required for recruiting HDAC and PcG proteins to the Arf locus. Our results are consistent with a model where p53 binds specifically to the Arf locus and then recruits HDAC to deacetylate the Arf locus. Deacetylation of the Arf locus then facilitates the recruitment of PRC to the Arf locus, leading to H3K27 trimethylation and silencing of Arf expression (Fig. 7C). This model is supported by 3 lines of evidence. First, Arf expression is elevated in vivo in multiple p53-deficient tissues or organs is rapidly elevated upon functional inactivation of p53 and is further repressed...
upon the activation of endogenous p53 in early passage of WT MEFs. Second, p53 directly binds to Arf locus, and that both transactivation and DNA binding activities of p53 are required for the repression of Arf and, importantly, for the binding to and repression of Arf by both HDAC and PRC. Third, we have also shown that both HDAC and PcG are conversely required for p53 to repress Arf expression.

The findings presented here shed mechanistic insights on the p53-mediated oncogenic checkpoint pathway: one on the mechanism of Arf activation and the other on the feedback regulation of p53. Although it has been observed for more than a decade that many hyperproliferative oncogenes can activate Arf expression (31), the molecular mechanism underlying oncogenic activation of Arf is unknown. Our demonstration that Arf is bound and repressed by p53 during normal cell growth suggests a critical step—dissociating p53 from the Arf locus—for an oncogene to activate Arf expression. It will be interesting to determine how an oncogene causes p53

Figure 6. HDAC function is required for PRC to bind Arf. A, WT p3 MEFs were treated with 0.1 μmol/L TSA for 24 hours. Cell pellets were collected and analyzed for binding of Ring1B on Arf by ChIP. B, WT p3 MEFs were treated with 0.1 μmol/L TSA or 10 mmol/L NaB for 30 hours and cells were collected and analyzed for Arf mRNA amount by Q-RT-PCR. C, cells in B were collected and analyzed for histone H4 acetylation, histone H3K27 trimethylation, and Ring1B binding on Arf locus by ChIP-Q-PCR. D, WT MEFs were infected with either an sh-luciferase (Luc) or shHDAC-expressing retovirus, selected by puromycin for 2 days, and collected 4 days after infection. The mRNA levels of HDAC1, HDAC2, and Arf were determined by Q-RT-PCR, and the binding of Bmi1 to Arf locus was determined by ChIP-Q-PCR.
dissociation in the presence of increased level of p53 since p53 activation would lead to p53 stabilization.

Feedback inhibition is a regulatory strategy commonly used in biochemical reactions such as the inhibition of threonine dehydrase by isoleucine (45). The accumulation of an end product inhibits the enzyme involved in its synthesis to avoid excessive accumulation and waste of resources. A similar strategy is also widely employed in cell regulations, especially those involved in cell growth and proliferation, to ensure a balanced homeostasis and cell physiology. Feedback inhibition is particularly needed for the control of the function of a gene, such as p53, whose activity, if not feedback inhibited, could lead to an irreversible consequence to the cell such as permanent cell-cycle arrest or cell death. At low, nonlethal levels of DNA damage, cell-cycle progression is delayed by the activation of p53 and then p21 to give cells time to repair the DNA and then resumed when the repair is completed. The resumption of cell-cycle progression is achieved through the p53–MDM2 feedback loop in which p53 activates the transcription of its primary inhibitor (6–8). ARF gene expression

Figure 7. HDAC and PcG function are required for p53 to repress Arf. A, WT p2 MEFs were treated with TSA and Nutlin-3 either alone or in combination for 24 hours. Cells were collected and analyzed for mRNA levels of p21 and Arf by Q-RT-PCR. B, WT MEFs (p2) were infected with either an sh-luciferase (Luc) or shBmi1-expressing retrovirus, selected by 2 μmol/L puromycin for 2 days, continuously cultured for 5 days after infection, and then treated with Nutlin-3 for 24 hours. Cells were collected and analyzed for Bmi1, p21, and Arf mRNA level by Q-RT-PCR. C, a schematic model illustrating p53-mediated Arf repression by HDAC and PcG. See text for more details.
exhibits a strong inverse correlation with the functional status of p53 in both human (19, 32) and mouse cells (33, 34), suggesting a possible feedback repression of ARF expression by p53. Our results provide a molecular basis supporting this feedback regulation. The significance of evolving this second feedback inhibition loop is that the first p53–MDM2 negative feedback loop would not be effective to inhibit p53 to resume the cell cycle if ARF expression is not repressed: the continuously synthesized ARF would bind to and prevent MDM2 from degrading p53 (Fig. 7C).

Our study also adds to the understanding of p53-mediated transcriptional repression, an area that is much less understood than p53-mediated transcriptional activation although an estimated 15% of genes containing a p53 response element can be repressed by p53 (see recent review in refs. 2, 46). p53 has been reported to directly bind with mSin3A, a transcriptional corepressor and a member of class I HDAC complexes and recruit HDACs to a specific promoter such as Mapl or Nanog (47, 48). We also confirmed the association between p53 and HDAC by detecting p53-HDAC1 binding in WT MEFs (Supplementary Fig. SD). Our study provides 2 separate lines of evidence supporting a role of histone deacetylation in p53-mediated repression of ARF. First, we showed that treatment of MEFs with a low concentration of TSA drastically increased ARF mRNA (>15-fold) and this effect is seen as early as 6 hours (Fig. 3). Second, we demonstrate that HDAC1 directly binds to and deacetylates ARF in a p53-dependent manner. We further identify a new mechanism—recruiting PRC—for p53-mediated transcriptional repression. To the best of our knowledge, this represents the first evidence that p53-mediated repression involves PRC which contains histone-modifying activities known to function in silencing gene expression. Conversely, identification of a sequence-specific binding factor—p53—in the recruitment of PRC to a specific locus also helps to better understand how PRC is recruited to their targets, a puzzling issue associated with the repression of many PRC-regulated genes in mammalian cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Yi Zhang, Ned Sharpless, and members of Xiong laboratory for the discussions throughout this study, Yizhou He for providing the retroviral construct targeting p53, and Dr. Zhi Liu for p53−/− mice.

Grant Support

This study was supported by NIH grant CA68377 to Y. Xiong. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 24, 2010; revised January 13, 2011; accepted February 7, 2011; published OnlineFirst March 29, 2011.

A Second Feedback Loop in p53 Inhibition


References


32. Robertson KD, Jones PA. The human ARF cell cycle regulatory gene promoter is a CpG island which can be silenced by DNA methylation and down-regulated by wild-type p53. Mol Cell Biol 1998;18:6457–73.


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Yaxue Zeng, Yojiro Kotake, Xin-Hai Pei, et al.

Cancer Res 2011;71:2781-2792. Published OnlineFirst March 31, 2011.

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